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Determination of the Inactivation Kinetics of Hepatitis A Virus in Human Plasma Products Using a Simple $TCID_{50}$ Assay

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The transmission of hepatitis A virus (HAV) associated with use of FVIII concentrates has been reported in a number of European countries. All of these cases were associated with products inactivated by use of solvent detergent treatment. These reports have emphasized the necessity of evaluating virus inactivation methodologies for their ability to inactivate HAV. Such studies had previously been hampered by the difficulties associated with titration of HAV, because of the minimal cytopathic effect of most strains of virus on tissue culture cells. We have developed a simple, rapid, $TCID_{50}$ virus titration system using a cytopathic strain of HAV which allows extensive kinetic studies of HAV inactivation. This has been compared with the standard radioimmunofocus forming (RFF) assay which is presently used for HAV titration. The reproducibility of the $TCID_{50}$ assay was demonstrated to be equal to that of the RFF assay and the 95% confidence intervals for titres determined by both assays were also equal. The thermal stability of the cytopathic strain was studied and shown to be equivalent to that of a noncytopathic strain. The kinetics of HAV inactivation by heating in aqueous solution were compared to those of HIV-1 and a number of model viruses. It was demonstrated that HAV was highly stable, with 5 hours heat treatment at 60°C in aqueous solution being required to inactivate 5.8 $\log_{10}$ virus. In contrast to heating in aqueous solution, lyophilization followed by 1 hour vapor heating at 60°C was sufficient to inactivate 5.9 $\log_{10}$ HAV.

KEY WORDS: HAV titration, cytopathic strain, vapor heating

INTRODUCTION

Hepatitis A is a widespread infectious disease that is endemic in developing countries, and that also may account for up to 25% of all cases of hepatitis in the developed world. Hepatitis A virus (HAV) is excreted in the feces of infected persons and was thought to spread exclusively via the fecal-oral route. Viraemia is known to occur for 2–4 weeks during the presymptomatic phase of HAV infection [Krugman et al., 1962; Lemon, 1994] and cases of hepatitis A transmission have been described that appear to have been caused by the treatment of patients with whole blood or plasma donations [Hollinger et al., 1983; Sheretz et al., 1984]. However, the transmission of virus by blood products such as clotting factor concentrates had been unknown until 1992 when over a hundred cases of hepatitis A transmission by Factor VIII concentrates in a number of European countries were reported [Mannucci, 1992; Gerritzen et al., 1992; Temperley et al., 1992; Peerlinck and Vermylen, 1993].

These findings have emphasized the necessity to develop methodologies capable of inactivating possible contaminating HAV in human blood products. These developments have been hampered by the difficulties associated with the slow rate of growth and the minimal cytopathic effects of most strains of HAV on infected tissue culture cells. The typical outcome of HAV infection of cell cultures is a protracted growth cycle with minimal effects on infected cells, leading to a persistent infection of the entire cell population with continuous production of infectious virus [Provost and Hilleman, 1979]. This had prevented the establishment of in vitro titration systems which would allow extensive kinetic studies of HAV inactivation. However, variants of HAV which display an accelerated growth phenotype in vitro have been selected by serial passage of the virus in cultured cells. After such prolonged passage of a virulent HAV strain HM-175 in primary cultures of African green monkey kidney cells (AGMK), virus variants which grew significantly more efficiently in these cells than did the wild-type virus were isolated [Daemer et al., 1981; Tedeschi et al., 1993]. A radioimmunofocus forming assay for quantitation of HAV in cell culture has been developed [Lemon et al., 1983], but this methodology, which

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requires the use of radioactive reagents, is labour intensive and time consuming. We have developed a simple, highly accurate $TCID_{50}$ titration procedure using a cytopathic strain of HAV, which allows extensive kinetic investigations of virus inactivation procedures. This procedure was then validated by comparison with the established radioimmunofocus methodology and the thermostability of both cytopathic and noncytopathic strains was also compared. The $TCID_{50}$ assay has now been used to determine the efficacy of two different virus inactivation methodologies in inactivating HAV in comparison to HIV-1 and model viruses.

MATERIALS AND METHODS

Cells and Viruses

A cytopathic HAV variant, HM 175/24a clone C, isolated and characterized as described in Cromeans et al. [1989] and Lemon et al. [1991] was used in these studies. This was kindly supplied by Dr. S. Emerson, NIAID, NIH, USA. The noncytopathic strain H2 std used in these studies was obtained by transfection of AGMK cells with RNA transcripts derived from the pHAV-7 plasmid (cDNA from HM-175, passage 35 [Cohen et al., 1987b]). In order to avoid multiple passaging of both viruses and possible changes in their cytopathogenicity, a master seed stock was prepared from one vial of virus and this was used to prepare an intermediate seed stock. This was then used to generate the working seed stock which was used for all experiments. Virus seed stocks were prepared in BS-C-1 cells using a master cell seed, working seed system as described above to ensure that cells between passage number 79 to 85 were used for preparation of all virus stocks. Stocks were prepared by infection of monolayers at a m.o.i. of 0.5 $TCID_{50}$/cell and were harvested following discarding of the cell medium and trypsinization of the monolayer. The trypsinized monolayer was pelleted, resuspended in one-tenth of the original medium volume, frozen and thawed before aliquoting and freezing at −80°C. This procedure gave reproducible high titre ($>10^8$ $TCID_{50}$/ml) virus stocks.

The Parvovirus, minute virus of mice (MVM) was grown and titrated on A-9 cells. HIV-1IIIB was grown on H9 cells and titrated on AA-2 cells. Tick-borne encephalitis virus (TBEV, strain Neudörfl) was grown on Vero cells and titrated on PS cells. Pseudorabies virus (PRV, strain Kaplan) was grown and titrated on Vero cells. All of these viruses with the exception of TBEV were titrated by standard $TCID_{50}$ methodology and the titre was calculated using logit analysis according to the maximum likelihood method [Cramer, 1964]. TBEV was titrated by standard plaque assay methodology.

HAV Titration

HM-175/24a was titrated on FRhK-4 cells (Fetal rhesus monkey kidney) by a standard $TCID_{50}$ methodology. The virus titre was determined following serial $\frac{1}{2}$ log dilution in tissue culture medium. One hundred microliters of each serial dilution was then added to each of eight wells of a microtitre plate containing 1×10^4 FRhK-4 cells per well. The plates were then incubated at 37°C for 14 days with a medium change after 7 days. After this incubation period the cytopathic effect (cpe) of the virus in each well was evaluated microscopically and the $TCID_{50}$ was determined on the basis of the number of wells displaying positive cpe. The 95% confidence intervals (C.I.) for each titration were calculated by the standard methodology [Cramer, 1964].

The radioimmunofocus forming assay (RFF) was carried out as described by Lemon et al. [1983]. Briefly, 0.5 ml of tenfold virus dilutions were added to FRhK-4 cell monolayers grown on coverslips in 60 mm petri dishes. After a 4 hour incubation, an agarose overlay was added and the petri dishes were incubated at 37°C. The overlays were removed at 10 days post infection, and the monolayers were fixed for 10 min at room temperature with acetone. A 0.1 ml volume of anti-HAV chimpanzee IgG (a gift from Dr. S. Emerson) was added to each monolayer and the coverslips were incubated at 37°C for 1 hour. The monolayers were washed once with PBS/Glycine/Tween-80 followed by a second incubation for 1 hour with I^{125} labelled anti-human IgG $F(ab)_2$. After washing four times with PBS/Glycine/Tween-80 the coverslips were air dried and exposed to Kodak XAR film with intensifier screen at −80°C for 3 days, and after development of the film, the radioimmunofocus forming units (RFU) were counted. The titre expressed as RFU/ml was determined according to the Poisson distribution.

RESULTS

Cytopathogenicity of the HM-175/24a Strain

The HM-175/24a strain gave a clearly visible cpe on FRhK-4 cells (Fig. 1a,b). This allowed microscopic evaluation of virus replication and enabled the establishment of a $TCID_{50}$ titration system. The enhanced cytopathogenicity of this strain was also demonstrated by an increased plaque size compared to the non-cytopathic strain H2 std using the radioimmunofocus assay (Fig. 1c,d).

Variance of the Titration System

The $TCID_{50}$ titre determination was validated by titration of a virus stock using this methodology and the radioimmunofocus assay on 5 consecutive days. The $TCID_{50}$ and RFU titres were determined as described in Methods and the 95% confidence interval was also calculated. The results are listed in Table I. Both assay systems gave highly reproducible results with titres ranging from $10^{8.2}$ to $10^{8.5}$ and from $10^{7.5}$ to $10^{7.7}$ per millilitre being obtained for the $TCID_{50}$ and RFF assays respectively. The 95% confidence intervals for the titre calculation obtained for the RFF assay were, however, narrower than those for the $TCID_{50}$ assay. The standard deviation of the values obtained on 5 consecutive days were calculated to be $10^{0.12}$ for the $TCID_{50}$ assay and $10^{0.10}$ for the RFF assay.

Comparison of the Inactivation Kinetics of Different HAV Strains

It has been reported that adaption of HAV to growth in cell culture is associated with the accumulation of multiple mutations and led to attenuation of virulence

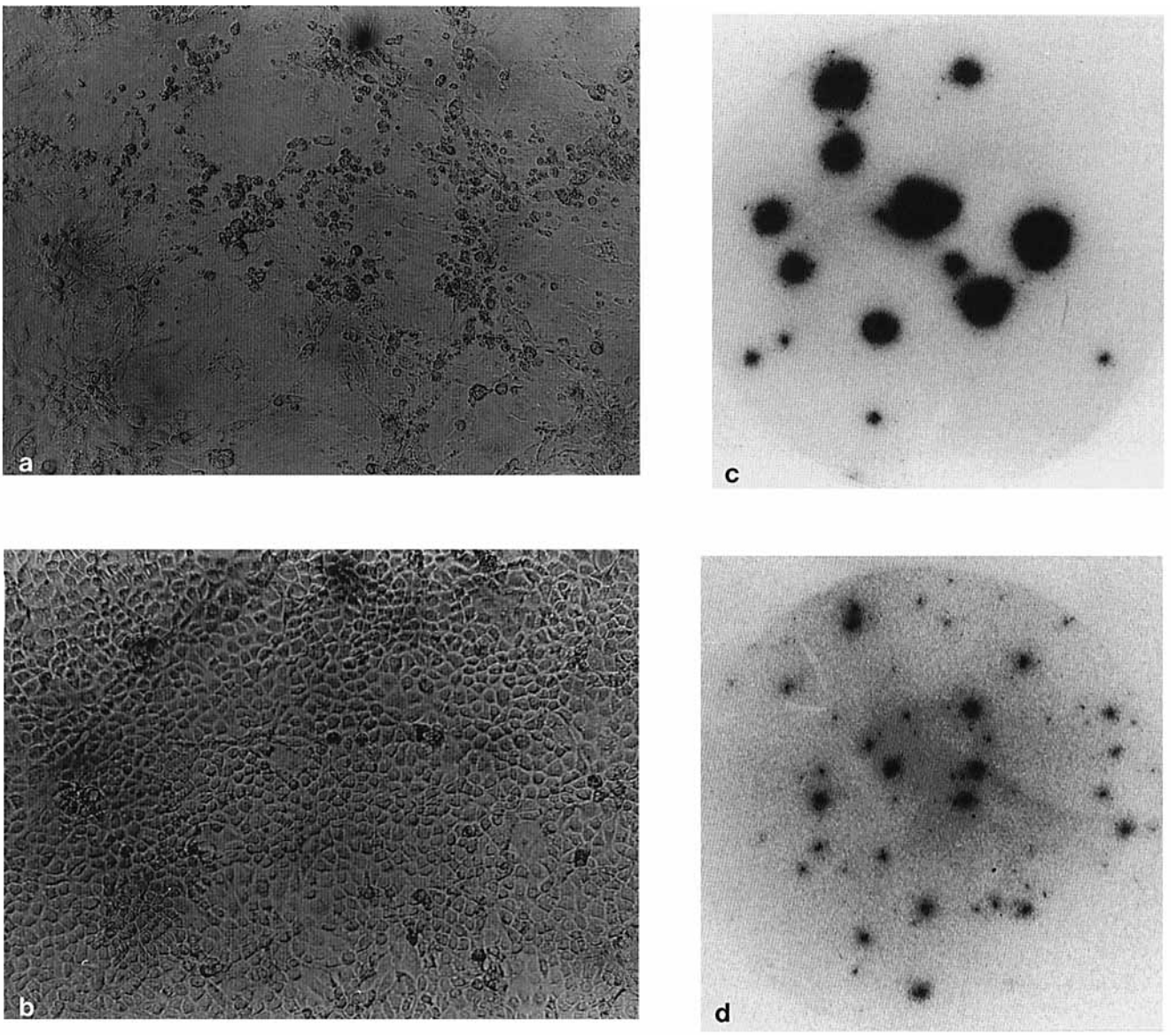

Fig. 1. **a:** Cytopathic effect of HM 175/24a, Clone C on FRhK-4 cells. **b:** Uninfected cells. **c,d:** Focus forming assay for HM 175/24a, Clone C cytopathic strain (c) and noncytopathic strain H2 std (d) on FRhK-4 cells.

TABLE I. Titration of Cytopathic Strain HM 175/24a, Clone C by $TCID_{50}$ Determination and Radioimmunofocus Forming (RFF) Assay

Replicate	$TCID_{50}$/ml (95% C.I.)	RFU/ml (95% C.I.)
1	$10^{8.4}$ ($10^{8.0}$–$10^{8.7}$)	$10^{7.7}$ ($10^{7.6}$–$10^{7.9}$)
2	$10^{8.2}$ ($10^{7.8}$–$10^{8.5}$)	$10^{7.5}$ ($10^{7.3}$–$10^{7.7}$)
3	$10^{8.4}$ ($10^{8.0}$–$10^{8.7}$)	$10^{7.7}$ ($10^{7.5}$–$10^{7.8}$)
4	$10^{8.5}$ ($10^{8.1}$–$10^{8.8}$)	$10^{7.7}$ ($10^{7.5}$–$10^{7.9}$)
5	$10^{8.2}$ ($10^{7.8}$–$10^{8.6}$)	$10^{7.5}$ ($10^{7.4}$–$10^{7.8}$)
Standard deviation	$10^{0.12}$	$10^{0.10}$

in marmosets and chimpanzees [Cohen et al., 1987a; Karron et al., 1988]. It is thus possible that the accumulation of mutations which led to generation of the cytopathic strain could lead to an alteration in the thermostability of the virus compared to the noncytopathic strain. Therefore the inactivation kinetics of the cytopathic and noncytopathic strains were compared using a standard virus inactivation methodology, i.e., heating in albumin solution at 60°C [Gellis et al., 1948]. Virus was added to a 5% human albumin solution and heated at 60°C for 10 hours. Samples were drawn at defined time intervals and frozen at −80°C prior to titration of both viruses by means of the RFF assay.

The data shown in Figure 2 represent the mean titres for each time point from duplicate experiments. These data indicate that both strains of HAV demonstrated practically identical inactivation kinetics with 4.7 $\log_{10}$ of the non-cytopathic strain H2 std and 4.6 $\log_{10}$ of the cytopathic strain HM-175/24a being inactivated after 10 hours of heat treatment.

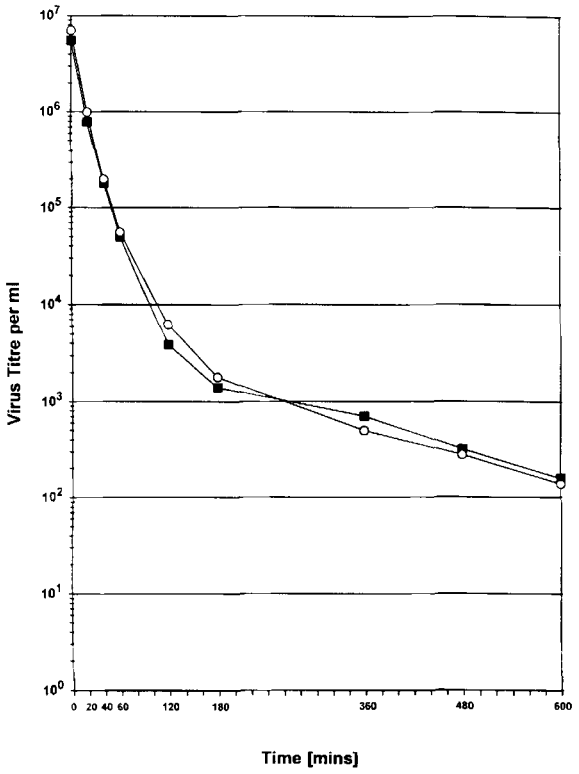

Fig. 2. Inactivation of the cytopathic (HM 175/24a, Clone C) and noncytopathic (H2 std) strains by heating at 60°C in a 5% human serum albumin solution. Circles represent the H2 std and squares the HM 175/24a, Clone C titres.

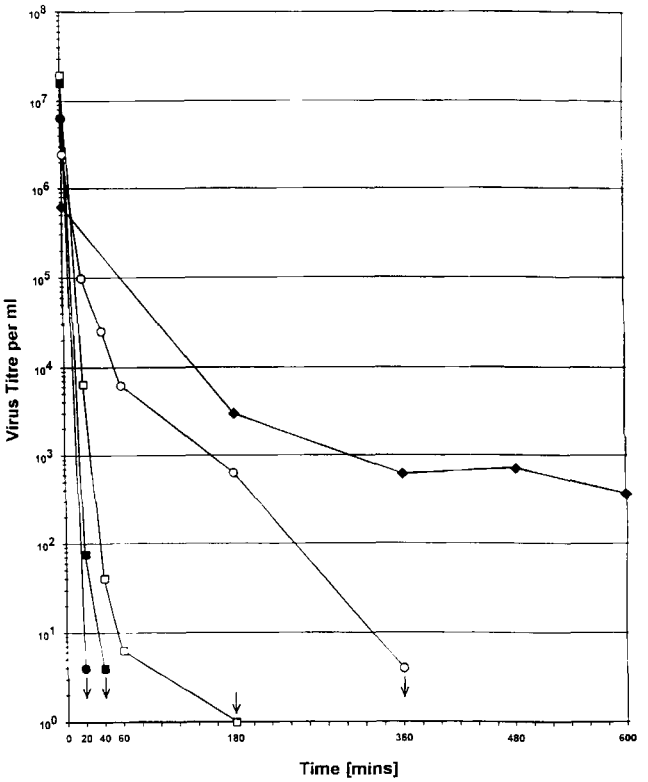

Fig. 3. Inactivation of HAV (open circles), HIV-1 (closed circles), TBEV (open squares), PRV (closed squares), and MVM (diamonds) by heating at 60°C in an AT-III aqueous solution. Arrows represent the limits of detection of the virus assay. Virus titres for HAV, HIV-1, PRV, and MVM are expressed as $TCID_{50}$/ml and for TBEV in PFU/ml.

Comparison of the Kinetics of Inactivation of HAV, HIV, and Model Viruses by Heating in Solution

Antithrombin III (AT-III) is a human plasma derived product used for prophylaxis and treatment of thrombotic and thromboembolic disorders. This is subjected to a viral inactivation procedure involving heat treatment in citrate buffer at 60°C for 10 hours [Holleman et al., 1977].

The inactivation kinetics of HAV were determined using this standard inactivation methodology and compared with those of HIV-1, TBEV (a model for hepatitis C virus), PRV (a model for lipid enveloped DNA viruses such as hepatitis B virus), and MVM (a model for B19 Parvovirus). These viruses are the most relevant target viruses which have been reported to be transmitted by human plasma products. Virus was diluted 1:10 in the antithrombin-III solution and samples were drawn for virus titration after various time intervals at 60°C.

The data in Figure 3 demonstrate that HAV is much more thermostable than HIV-1 and TBEV with 5 hours at 60°C being required to inactivate 5.8 $\log_{10}$ of this virus. In comparison 6.2 $\log_{10}$ HIV-1, 6.6 $\log_{10}$ PRV and 7.3 $\log_{10}$ TBEV were inactivated within 20 minutes, 40 minutes, and 3 hours respectively. The only test virus which proved to be more thermostable than HAV was MVM, with only 3.2 $\log_{10}$ being inactivated by 10 hours of heat treatment.

HAV Inactivation by Vapor Heating

Virus inactivation by vapor heating of human plasma derived products such as coagulation factors is a well established methodology [Kurth et al., 1986; Dorner et al., 1993]. This methodology involves lyophilization, followed by addition of water to the lyophilized powder to achieve homogeneous moistening. This is followed by a heating procedure at 60°C for 10 hours followed by 80°C for 1 hour in an airtight container under a nitrogen gas atmosphere. This procedure was validated for its efficacy against a range of viruses, i.e., HAV, HIV-1, TBEV, PRV, and MVM in a Factor VII concentrate. Virus was added to the solution, which was then lyophilized, moistened, and heated at 60°C for 10 hours and then at 80°C for 1 hour. Samples were drawn before and after lyophilization and at various time intervals at 60°C and 80°C. Lyophilized samples were resuspended for titration in the original volume before lyophilization. The data in Table II demonstrate that HAV was readily inactivated by vapor heating with 5.9 $\log_{10}$ being inactivated after lyophilization and 1 hour at 60°C. In comparison, 6 hours of inactivation were required to inactivate 6.6 $\log_{10}$ TBEV and 3 hours to inactivate 6.2 $\log_{10}$ HIV-1 and 6.9 $\log_{10}$

TABLE II. Comparison of the Kinetics of Inactivation by Vapor Heating of HAV, HIV-1, and Model Viruses in a FVII Concentrate (Virus Titers in $TCID_{50}$/ml and for TBEV in PFU/ml)

Virus	Titre before lyophilization	Time at 60°C (hr)					
		0	1	3	6	10	10 + 1/hr, 80°C
HAV	$10^{6.5}$	$10^{3.6}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$
HIV-1	$10^{6.8}$	$10^{4.9}$	$10^{1.3}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$
TBEV	$10^{6.6}$	$10^{6.2}$	$10^{1.8}$	$10^{0.7}$	$<10^{0}$	$<10^{0}$	$<10^{0}$
PRV	$10^{7.5}$	$10^{4.1}$	$10^{0.9}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$	$<10^{0.6}$
MVM	$10^{6.7}$	$10^{5.7}$	$10^{5.7}$	$10^{5.5}$	$10^{4.6}$	$10^{4.5}$	$10^{1.9}$

PRV. The Parvovirus MVM was extremely stable with 10 hours at 60°C followed by 1 hour at 80°C being required to inactivate 4.8 $\log_{10}$ of this virus.

DISCUSSION

The validation of virus inactivation procedures with HAV has proved difficult because of the problems associated with the use of an assay system requiring radioactive reagents. Therefore we have attempted to establish a methodology which would allow titration of HAV with a simple nonradioactive assay which gives reproducible results with low fiducal limits. The data presented in Table I demonstrate that the $TCID_{50}$ assay fulfils all of these requirements. Five replicate titrations gave titres ranging from $10^{8.2}$ to $10^{8.5}$ $TCID_{50}$/ml with a calculated S.D. of 0.12. The RFF titres for the same virus stock ranged from $10^{7.5}$ to $10^{7.7}$ RFU/ml with a S.D. of 0.10.

Even when these titres are converted to $TCID_{50}$ by multiplication with the Poisson coefficient 0.69, the titres obtained by the $TCID_{50}$ assay are slightly higher than those obtained for the RFF assay. This might indicate that this system is more sensitive than the RFF assay but it could also be explained by the fact that the RFF assay involves virus replication for 10 days while the $TCID_{50}$ assay is carried out for 14 days. The reproducibility of both assays is approximately equal with the S.D. obtained for the RFF assay being a little lower. However, the main advantage of this $TCID_{50}$ methodology compared to the RFF assay is the simplicity of the assay with no manipulations being required after titration other than a medium change after 7 days. Another simplified $TCID_{50}$ assay for HAV has been published [Hart et al., 1994] but this methodology still requires use of a sandwich ELISA to detect HAV antigen after a 14-day incubation period. This has the disadvantage of being more labour intensive and possibly less clearcut in that a relatively high absorbance value of ≥ 0.3 is chosen as a cut-off to eliminate the occasional edge effects of ELISA methodology.

The thermal stability characteristics of the cytopathic strain were compared with those of the noncytopathic strain to ensure that inactivation data obtained using the cytopathic strain were relevant with respect to blood product contamination with the wild-type virus. There was no difference in the inactivation kinetics of the two strains following heating at 60°C in a 5% human albumin solution. This confirms the findings of Lemon et al. [1991], who demonstrated no reproducible differences in the thermal stability of a noncytopathic HAV and two cytopathic clones at a range of temperatures. This demonstrates clearly that validation studies for blood product safety with respect to HAV transmission can be carried out with the cytopathic strain. The availability of a simple titration system for this strain now facilitates the extensive validation studies which are required by regulatory authorities.

We have carried out such preliminary studies using two different inactivation procedures, i.e., heating an AT-III solution at 60°C and vapor heating of a moistened lyophilized F VII powder at 60°C and 80°C. The data presented in Figure 3 demonstrate that HAV was extremely stable in AT-III solution, compared to HIV-1 and the model viruses TBEV and PRV. Five hours were required to inactivate 5.8 $\log_{10}$ of HAV while 6.2 $\log_{10}$ HIV-1 were inactivated after 20 minutes heat treatment. HAV was, however, inactivated more quickly in AT-III solution than in a 5% albumin solution (Fig. 2) at the same inactivation temperature. This emphasizes that the rate of inactivation is dependent not only on the temperature but on protein composition and concentration and the presence of stabilizers in the buffer.

The vapor heating procedure which involves lyophilization followed by heat treatment of the moistened lyophilizate was highly effective in inactivating HAV. This was partially due to the high loss of titre i.e. 2.9 $\log_{10}$ obtained by lyophilization. This is, however, in agreement with the observations of Lemon et al. [1994] who reported a loss of titre of 2.3 $\log_{10}$ following lyophilization of HAV in a factor VIII concentrate. Following lyophilization, vapor heating of FVII concentrate for 1 hour at 60°C reduced the HAV titre from $10^{3.6}$ to below the level of detection. The rate of inactivation of HAV by vapor heating was more rapid than that for HIV-1, TBEV and PRV and particularly for the Parvovirus MVM, which is also a small nonlipid enveloped virus. Ten hours at 60°C followed by 1 hour at 80°C were required to inactivate 4.8 $\log_{10}$ of this highly stable virus. This rapid rate of HAV inactivation is, however, partially due to the sensitivity of HAV to lyophilization.

These data indicate that vapor heating in particular has the potential to inactivate very high titres of HAV. Although it is not possible to estimate the maximum titre of HAV which could be present in a plasma pool or plasma product, the use of vapor heating at 60°C for 10

hours plus 1 hour at 80°C provides an extremely high safety margin in that lyophilization plus 1 hour at 60°C alone is sufficient to inactivate 5.9 $\log_{10}$ HAV in a FVII concentrate. These studies are now being extended to a range of coagulation factors and other plasma products to determine the efficacy of vapor heating and other inactivation methodologies in inactivating hepatitis A virus.

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